Queensland Health

HealthSupport Queensland

Project #182

Validation of 3500xL Analysis of Casework PowerPlex®21 WEN

June 2016

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Queensland Government

Document Details

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Version history

Version	Date	Changed by	Description
1.0	June 2015	Biljana Micic, Megan Mathieson, Luke Ryan	Document Created

Document sign off

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1. Abstract

Forensic DNA Analysis currently uses the 3130*xl* Genetic Analysers (Life Technologies, Applied Biosystems, Foster City, CA, US) for capillary electrophoresis analysis of casework samples amplified with PowerPlex®21. The 3130*xl* instruments have been superseded by the 3500 Series Genetic Analyser. Forensic DNA Analysis has two 3500xL Genetic Analysers, which have been successfully validated for the analysis of reference samples amplified with PowerPlex®21 both for extracted reference[1] and direct amplification[2].

A validation of the 3500xL for the analysis of PowerPlex®21 for casework samples was conducted previously in 2015[3]. Saturation and mixture studies were not completed during this validation due to the quality of the mixture samples due to poor spectral separation for PowerPlex®21 produced on the 3500xL. The results from this validation show the 3500xL Genetic Analyzer instruments are unsuitable to use for the analysis of extracted casework samples amplified using PowerPlex®21 at this time. Although the mixture studies resulted in this validation not being accepted, other results from this project were accepted for possible future implementation should the spectral separation be resolved. This validation was conducted using the CC5 PowerPlex®21 internal lane standard (ILS).

Promega (PowerPlex®21 manufacturers) have advised that they have modified the PowerPlex®21 System, and the current formulation for the system will be replaced by the new formulation in June 2016[4]. In the new formulation of the PowerPlex®21 System the Internal Lane Standard (ILS) and the PowerPlex®5-Dye Matrix standards are being modified. The new ILS has a different dye (WEN) to what is in the current ILS (CC5 dye). Because the dye used for the ILS is being changed, a corresponding dye change is being made to the PowerPlex®5-Dye Matrix standards. The matrix standards makeup is also being changed so the 5 matrix dyes will be combined into one tube, whereas currently they are provided in 5 separate tubes which are combined in-house.

Promega have advised the WEN dye has a stronger emission and has improved photostability when compared to CC5. The WEN ILS has the same fragment sizes and dye colour as CC5. In addition to kit formulation changes, Promega will also provide updated panels and bins. These are required because the new ILS with WEN dye migrates different to the ILS with CC5 dye[4].

PowerPlex®21 WEN has been validated and implemented for the following purposes[5]:

- Casework samples amplified with PowerPlex®21 analysed on the 3130*xl*
- FTA reference samples amplified with PowerPlex®21 by direct amplification analysed on the 3500xL

- Extracted reference sampled amplified with PowerPlex®21 analysed on the 3500xL.

Following notification from Promega of the changes to the PowerPlex®21 formulation an assessment of the 3500xL analysis of casework PowerPlex®21 WEN was conducted[6]. Baseline calculations and mixture studies were conducted to determine if the formulation change had resolved the spectral separation issues seen in the mixture studies in the original casework 3500xL PowerPlex®21 CC5 validation[3]. The assessment of the new PowerPlex®21 WEN formulation and dye matrix determined that the spectral separation issues have been resolved sufficiently to enable mixture interpretation.

This report will collate the results from previous casework 3500xL and PowerPlex®21 validations into one over-arching validation document a recommend the implementation of the 3500xL for the analysis of extracted casework samples, amplified with PowerPlex®21 WEN.

1 Results and Data Compilation

1.1. Baseline, Limit of Detection and Limit of Reporting

The baseline, limit of detection (LOD) and limit of reporting (LOR) was calculated in Project #177, the assessment of the PowerPlex®21 WEN for analysis of casework samples on the 3500xL [6].

The sample set was comprised of 10 CTS samples and 62 extraction negative controls. Based on the average quantification result, for each of the 10 CTS samples, a dilution series was created as per Table 1 below.

Table 1 – Baseline CTS Samples – Total DNA Quantity (ng)

#	Total DNA (ng)
1	0.700
2	0.600
3	0.500
4	0.447
5	0.394
6	0.342
7	0.289
8	0.236
9	0.183
10	0.131
11	0.078
12	0.025

Table 2 below shows the results of the baseline calculations for each of the individual dyes. Table 3 shows the results for the baseline calculations averaged across all sample dyes (i.e. excluding WEN) and also averaged across all dyes (i.e. sample dyes and WEN).

Table 3 - Baseline results for individual dyes

	Fluorescein (Blue)	JOE (Green)	TMR-ET (Yellow)	CXR-ET (Red)	WEN (Orange)
Min RFU	1	1	1	1	1
Max RFU	73	79	123	76	94
Average	3.5994	7.0152	11.9395	9.9641	3.3470
SD	2.2587	3.3723	5.0577	4.3883	2.9255

3SD	6.7760	10.1169	15.1730	13.1649	8.7765
10SD	22.5866	33.7231	50.5767	43.8830	29.2549
LOD (Ave+3SD)	**10.3754**	**17.1322**	**27.1125**	**23.1290**	**12.1235**
LOR (Ave+10SD)	**26.1861**	**40.7384**	**62.5161**	**53.8471**	**32.6019**

Table 4 - Baseline Results Averaged

	All Sample Dyes	**All Dyes (including WED)**
Min RFU	1	1
Max RFU	123	123
Average	7.7656	6.9256
SD	4.7272	4.7678
3SD	14.1817	14.3033
10SD	47.2722	47.6775
LOD (Ave+3SD)	**21.9473**	**21.2289**
LOR (Ave+10SD)	**55.03784**	**54.60314**

The lowest sample dye average peak height (3.5994RFU), LOD (10.3754RFU) and LOR (26.1861RFU) were all in the Fluorescein (Blue) sample dye. The highest sample dye average peak height (11.9395RFU), LOD (27.1125RFU) and LOR (62.5161RFU) were all in the TMR-ET (Yellow) sample dye.

Acceptance Criteria

Given that the baseline has not been previously calculated for the PowerPlex

1.2. Stutter

Stutter thresholds are an amplification artefacts usually observed as a peak one or two repeat units smaller in size than the true allele peak (-1 and -2 repeat stutter), or one repeat unit larger in size that the true allele peak (+1 repeat stutter). As stutter is an amplification artefact, it is not affected by a change in the size standard from CC5 to WEN, or a change in the dye matrix. Therefore the stutter results from the

PowerPlex®21 CC5 3500xL casework validation[3] are valid and can be adopted for implementation.

Table 10 shows the number of times stutter was observed, the average stutter ratio, standard deviation, and stutter threshold for -2 repeat, -1 repeat and +1 repeat stutter for each locus.

For the -2 repeat stutter, thresholds were calculated for 19 out of 20 loci. From these, 15 out of 20 had lower thresholds, 4 out of 20 had greater thresholds, and 1 out of 20 had the same thresholds as the current 3130*xl* thresholds. Due to no observations of -2 repeat stutter at Penta D stutter thresholds were unable to be calculated. The -2 repeat stutter thresholds calculated for the 3500xL are the recommended analysis thresholds for implementation as there are no recommended -2 repeat stutter thresholds from Promega.

For -1 repeat stutter, where the calculated thresholds are lower than the Promega PowerPlex®21 GeneMapper stutter threshold, the Promega PowerPlex®21 GeneMapper stutter threshold will be implemented as the analysis threshold. Where the calculated stutter threshold is greater than the Promega PowerPlex®21 stutter threshold the calculated stutter threshold will be implemented as the analysis threshold. For the -1 repeat stutter, 7 out of 20 loci had higher stutter thresholds, 12 had lower stutter thresholds and one was the same as the Promega stutter thresholds.

For the +1 repeat stutter, thresholds were calculated for all loci. Of these 17 of the 20 had lower thresholds, 3 out of 20 had greater thresholds than the current 3130*xl* thresholds. The +1 repeat stutter thresholds calculated for the 3500xL are the recommended analysis thresholds for implementation as there are no recommended +1 repeat stutter thresholds from Promega.

Table 11 shows the stutter thresholds for -2 repeat, -1 repeat and +1 repeat stutter calculated for the 3500xL, the -1 repeat stutter thresholds for PowerPlex®21 from Promega and the current thresholds used for the 3130*xl*.

Table 4 - Summary of Stutter Data

	-2 STUTTER					-1 STUTTER					+1 STUTTER				
Locus	No. Observed	Average	Standard Deviation	New 3500xL Casework Threshold %	3130*x*/ Current Casework Threshold %	No. Observed	Average	Standard Deviation	New 3500xL Casework Threshold %	3130*x*/ Current Casework Threshold %	No. Observed	Average	Standard Deviation	New 3500xL Casework Threshold %	3130*x*/ Current Casework Threshold %
D3S1358	90	0.0082	0.0039	2.0	2.3	263	0.0864	0.0141	12.9	14.2	127	0.0080	0.0064	2.7	4.3
D1S1656	118	0.0113	0.0102	4.2	4.3	627	0.0827	0.0219	14.9	17.2	300	0.0128	0.0060	3.1	6.7
D6S1043	62	0.0078	0.0032	1.8	2.7	574	0.0690	0.0135	10.9	12	305	0.0127	0.0061	3.1	7.4
D13S317	20	0.0065	0.0038	1.8	3.4	382	0.0483	0.0206	11.0	11.8	156	0.0141	0.0070	3.5	7.4
Penta E	2	0.0155	0.0000	1.6	2.1	510	0.0351	0.0155	8.2	10.7	24	0.0157	0.0087	4.2	1.7
D16S539	108	0.0066	0.0063	2.5	2.1	287	0.0659	0.0173	11.8	12.1	294	0.0109	0.0048	2.5	4.3
D18S51	126	0.0087	0.0054	2.5	2.8	497	0.0809	0.0226	14.9	16	272	0.0109	0.0093	3.9	4.9
D2S1338	178	0.0082	0.0037	1.9	2.9	627	0.0840	0.0183	13.9	14.9	45	0.0159	0.0113	5.0	6.5
CSF1PO	28	0.0050	0.0013	0.9	3.7	201	0.0592	0.0142	10.2	13.7	161	0.0107	0.0053	2.7	4.4
Penta D	0	0.0000	0.0000	0.0	0	326	0.0183	0.0067	3.8	8.2	8	0.0143	0.0094	4.3	8.8
TH01	104	0.0071	0.0040	1.9	2.6	499	0.0222	0.0094	5.0	8.7	67	0.0162	0.0116	5.1	2.1
vWA	18	0.0107	0.0088	3.7	3.5	280	0.0659	0.0274	14.8	15.2	39	0.0189	0.0135	5.9	5.6
D21S11	95	0.0188	0.0204	8.0	4	510	0.0831	0.0173	13.5	14.1	272	0.0157	0.0102	4.6	7.1
D7S820	33	0.0140	0.0102	4.5	3.4	354	0.0500	0.0208	11.3	11.4	81	0.0100	0.0061	2.8	5.8
D5S818	3	0.0132	0.0024	2.1	2.3	216	0.0534	0.0168	10.4	12	144	0.0117	0.0060	3.0	5.6
TPOX	3	0.0037	0.0011	0.7	2.7	435	0.0273	0.0104	5.9	9	18	0.0163	0.0095	4.5	6.3
D8S1179	122	0.0062	0.0021	1.3	2.6	394	0.0708	0.0149	11.5	13.2	257	0.0086	0.0046	2.2	5.5
D12S391	219	0.0121	0.0050	2.7	2.8	619	0.0949	0.0303	18.6	18.8	129	0.0109	0.0065	3.0	5.3
D19S433	121	0.0067	0.0041	1.9	3.2	377	0.0693	0.0154	11.5	12.8	35	0.0097	0.0076	3.3	7.1
FGA	105	0.0090	0.0035	1.9	3.1	457	0.0697	0.0180	12.4	13.8	168	0.0083	0.0046	2.2	5.9

Table 5 - Comparison of stutter thresholds between 3130*xl* and 3500xL

	-2 STUTTER		-1 STUTTER			+1 STUTTER	
Locus	New 3500xL Casework Threshold %	Current Casework Threshold %	New 3500xL Casework Threshold %	Promega Stutter File	Current Casework Threshold %	New 3500xL Casework Threshold %	Current Casework Threshold %
D3S1358	2.0	2.3	12.9	14.0	14.2	2.7	4.3
D1S1656	4.2	4.3	14.9	15.0	17.2	3.1	6.7
D6S1043	1.8	2.7	10.9	14.0	12.0	3.1	7.4
D13S317	1.8	3.4	11.0	11.0	11.8	3.5	7.4
Penta E	1.6	2.1	8.2	10.0	10.7	4.2	1.7
D16S539	2.5	2.1	11.8	12.0	12.1	2.5	4.3
D18S51	2.5	2.8	14.9	16.0	16.0	3.9	4.9
D2S1338	1.9	2.9	13.9	16.0	14.9	5.0	6.5
CSF1PO	0.9	3.7	10.2	11.0	13.7	2.7	4.4
Penta D	0.0	0.0	3.8	9.0	8.2	4.3	8.8
TH01	1.9	2.6	5.0	6.0	8.7	5.1	2.1
vWA	3.7	3.5	14.8	14.0	15.2	5.9	5.6
D21S11	8.0	4.0	13.5	13.0	14.1	4.6	7.1
D7S820	4.5	3.4	11.3	11.0	11.4	2.8	5.8
D5S818	2.1	2.3	10.4	10.0	12.0	3.0	5.6
TPOX	0.7	2.7	5.9	7.0	9.0	4.5	6.3
D8S1179	1.3	2.6	11.5	12.0	13.2	2.2	5.5
D12S391	2.7	2.8	18.6	17.0	18.8	3.0	5.3
D19S433	1.9	3.2	11.5	11.0	12.8	3.3	7.1
FGA	1.9	3.1	12.4	12.0	13.8	2.2	5.9

- Accepted Stutter Thresholds

1.1.1 Acceptance Criteria – Stutter Thresholds

Stutter is an amplification artefact and is not caused by capillary electrophoresis, therefore the stutter thresholds calculated using Powerplex®21 CC5 are validation for implementation with Powerplex®21 WEN.

Variation in stutter thresholds between the 3130*xl* and 3500xL are likely the result of amplification variation and/or differences in the size and composition of the data sets. Further, as stutter thresholds are a ratio of stutter peak height to allele peak height, they are not impacted by the peak height scale variation between the 3130*xl* and 3500xL.

The 3500xL stutter thresholds were generally comparable to the 3130*xl* thresholds and those in the Powerplex®21 stutter file. The stutter thresholds calculated for the 3500xL must be accepted and implemented, with a recommendation that these are reviewed post implementation using a larger data set for increased robustness.

1.3. Peak Height Ratio

Peak height ratio (PHR) is the ratio between the two peaks in a heterozygous pair. Under optimal conditions the amplification of a pair of alleles should result in equal peak heights however, input DNA, inhibitors and quality of DNA will affect the amplification. Amplification efficiency is the primary factor influencing the PHR, and not capillary electrophoresis or a change in size standard from CC5 to WEN. Therefore the PHR calculated in the PowerPlex®21 CC5 3500xL casework validation[3] is valid and can be adopted.

A total of 521 samples were used to calculate the peak height ratio. Table 6 summarises the results of the average PHR and allelic imbalance threshold (AI_{TH}) data calculated for all loci in the PowerPlex®21 system. All loci displayed high peak balances within each locus. The lowest observed PHR was at CSF1PO with 82% and a standard deviation of 0.1074, while the overall average PHR is 85% with a standard deviation of 0.1058.

Table 6 - Data of the PHR and calculated AI

	Average	SD	3 SD	AI_{TH}	n*
AMEL	0.8612	0.0972	0.2917	0.5695	392
D3S1358	0.8594	0.1061	0.3184	0.5411	81
D1S1656	0.8679	0.0949	0.2848	0.5831	307
D6S1043	0.8569	0.1053	0.3160	0.5408	256
D13S317	0.8374	0.1173	0.3518	0.4855	160
Penta E	0.8316	0.1180	0.3541	0.4776	344
D16S539	0.8719	0.1027	0.3082	0.5637	98
D18S51	0.8434	0.1038	0.3113	0.5321	231
D2S1338	0.8385	0.1117	0.3351	0.5034	284
CSF1PO	0.8202	0.1074	0.3223	0.4979	32
Penta D	0.8526	0.1005	0.3015	0.5511	174
TH01	0.8773	0.0806	0.2417	0.6356	226
vWA	0.8421	0.1056	0.3169	0.5252	135
D21S11	0.8546	0.1005	0.3014	0.5531	238
D7S820	0.8500	0.1066	0.3197	0.5354	136
D5S818	0.8305	0.1021	0.3063	0.5421	52
TPOX	0.8439	0.1177	0.3532	0.4907	155
D8S1179	0.8554	0.1002	0.3005	0.5549	161
D12S391	0.8438	0.1110	0.3330	0.5107	295
D19S433	0.8605	0.1115	0.3346	0.5259	137
FGA	0.8508	0.1099	0.3296	0.5212	231
All Loci	0.8514	0.1058	0.3175	0.5339	4125

*n = number of times peak height ratio was calculated for a locus

Figure 1 graphically shows the average PHR and the allelic imbalance across all loci. Using Equation 8 in Section 5.4 of the casework PowerPlex®21 3500xL validation[3] the overall allelic imbalance threshold (AI_{TH}) was calculated to be 53%. This is 8% higher than the threshold previously calculated for the 3130*xl*.

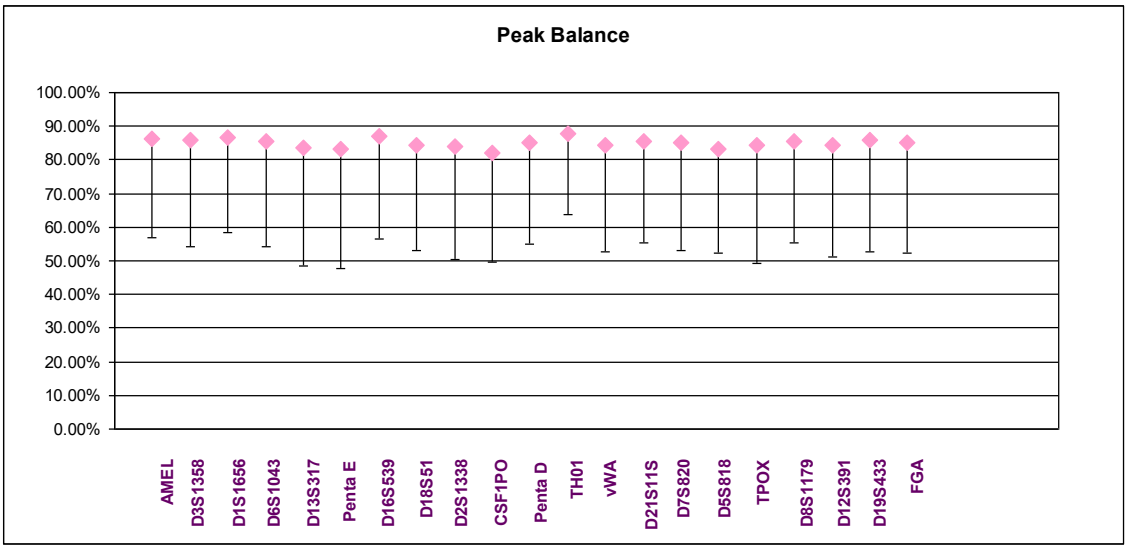

Figure 1 - Average Peak Height Ratios per locus

Note: error bars represent the mean PHR minus three times standard deviation

1.1.2 Acceptance Criteria – Allelic Imbalance Threshold

The ratio of peaks in a heterozygous pair (PHR) is primarily influenced by amplification and not capillary electrophoresis and/or the size standard. Therefore the PHR calculated in the PowerPlex®21 CC5 3500xL casework validation[3] is valid and can be adopted.

Variation in the AI_{TH} between the 3130*xl* and 3500xL is likely due to amplification variation and/or differences in the data sets used, rather than instrument factors. Further, as the AI_{TH} is a ratio of peak heights in a heterozygous pair, it is not impacted by the peak height scale variation between the 3130*xl* and 3500xL.

The 3500xL AI_{TH} for casework samples was calculated as 53%. The current AI_{TH} for casework samples on the 3130*xl* is 45%, which is comparable to the 3500xL threshold and therefore the 3500xL passes this experiment.

The AI_{TH} for casework samples will be accepted and rounded to 55% for implementation purposes.

1.4. Homozygote Peak Threshold

The homozygote threshold (Th_{Hom}) is the threshold above which you can be confident that a heterozygote locus will not be incorrectly called as a homozygote locus. The Th_{Hom} was calculated using equation 9 in Section 5.5 of the casework PowerPlex®21 3500xL validation[3].

In the Th_{Hom} calculation the AI_{TH} and LOR from this report (sections 1.3 and 1.1 respectively) were used.

The Homozygote peak threshold was calculated to be XXX RFU.

1.1.3 Acceptance Criteria – Homozygote Peak Threshold

For casework interpretation, the homozygote peak threshold is used only as a guide there are no acceptance criteria for this experiment. The Homozygote Peak Threshold will be accepted as XXX and rounded to XXX for implementation.

1.5. Repeatability and Reproducibility

Repeatability and Reproducibility experiments were performed in the PowerPlex®21 CC5 3500xL casework validation[3]. Given that the verification of PowerPlex®21 WEN[5] has shown that the new PowerPlex®21 WEN formulation accurately sizes DNA fragments (as per the CC5 ILS), the results of the repeatability and reproducibility experiment in the PowerPlex®21 CC5 3500xL casework validation[3] can be adopted.

Five samples were selected and run in quintuplicate (see Section 5.1.3[3] for plate map and sample set). Complete and concordant profiles were obtained from all the samples on all runs for repeatability and reproducibility testing on both the 3130*xl* and 3500xL. The peak height data from each run was compared by calculating the percentage change and performing a Student's t-test.

Repeatability is an assessment of the ability of the 3500xL with PowerPlex®21 to produce the same results when one sample set is processed a number of times by one user, under the same conditions.

Figure 2 shows the results of the repeatability testing on the 3130*xl*. The results show that the majority of the run to run variation of peak heights range from 6% to -22%. Two samples showed a significant difference (p = 0.0036 and 0.0419) in peak heights between run 1 and run 2. Peak heights on run 1 were higher than run 2 which may be due to run to run variation. For all other samples there was no significant difference ($p \geq 0.05$) in peak heights between run 1 and 2.

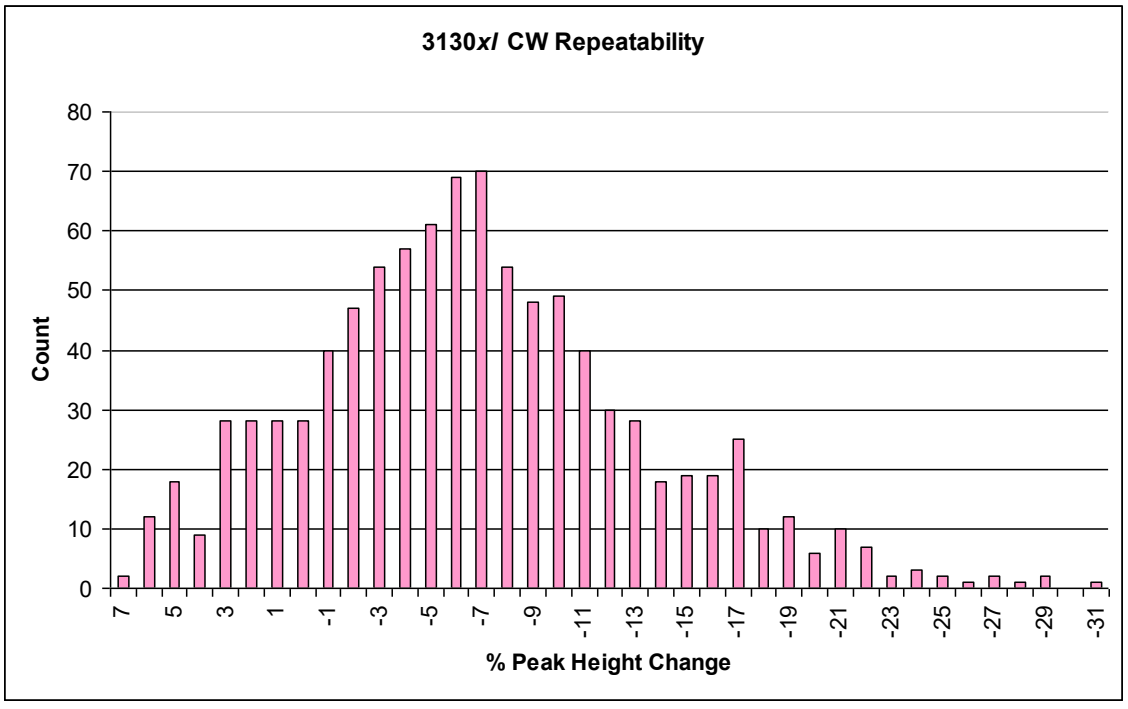

Figure 2 - 3130*xl* CW Repeatability

Figure 3 shows the results of the repeatability testing on the 3500xL. The results show the majority of the run to run variation of peak heights range from -3% to -18%. For all samples, there were no significant differences ($p \geq 0.05$) in peak heights between run 1 and 2.

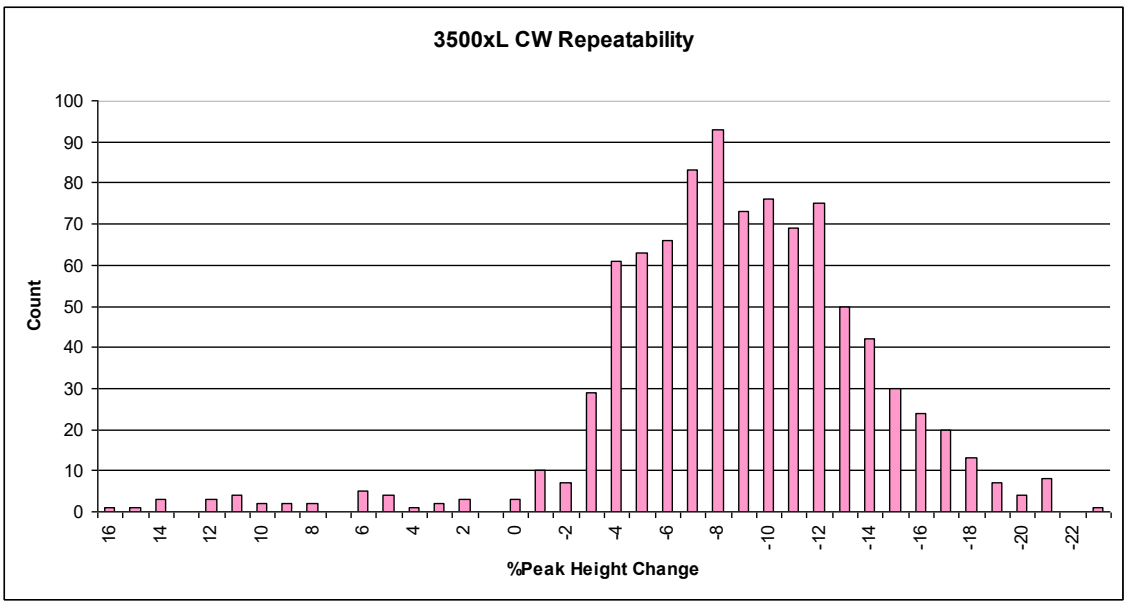

Figure 3 - 3500xL CW Repeatability

Reproducibility is an assessment of the ability of the 3500xL with PowerPlex®21 to produce the same results when one sample set is processed by different operators under different conditions.

Figure 4 shows the results of the reproducibility testing on the 3130*xl*. The results show that the majority of the run to run variation of peak heights range from 0% to -30%. Five samples showed a significant difference (p = 0.0484, 0.0090, 0.0153, 0.0054 and 0.0031) in peak heights between run 1 and run 2. Peak heights on run 1 were higher than run 2 which may be due to run to run variation. For all other samples there was no significant difference ($p \geq 0.05$) between run 1 and 2.

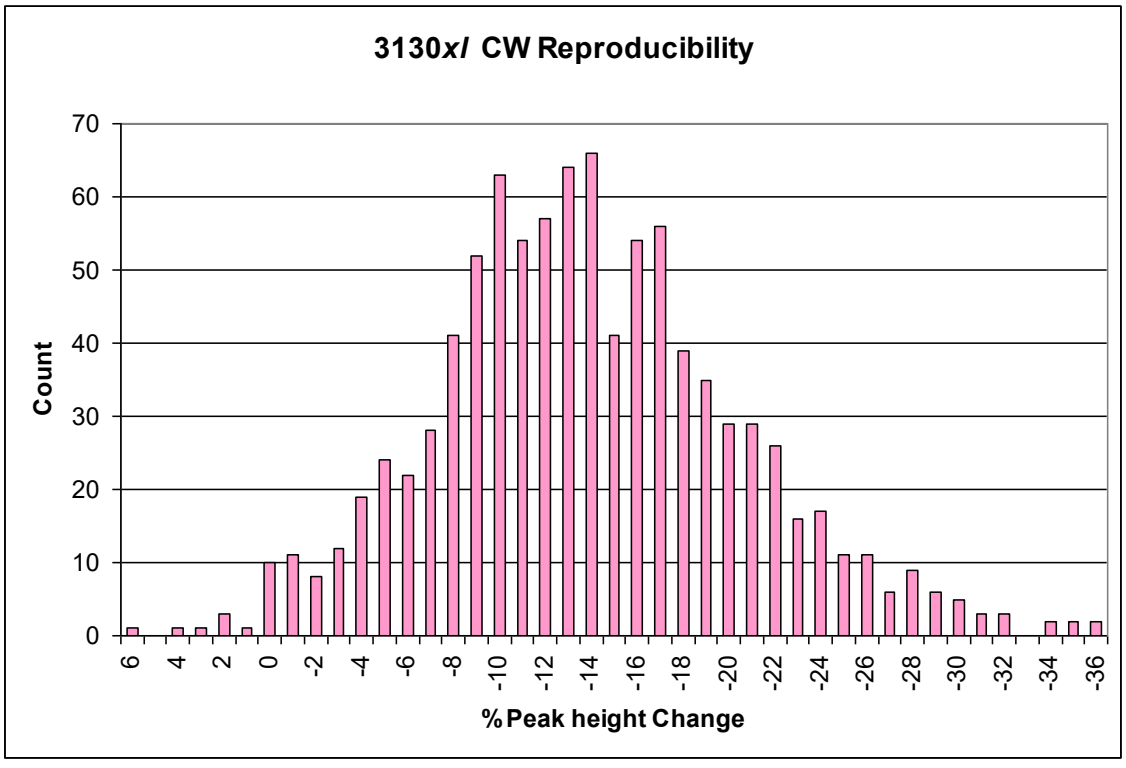

Figure 4 - 3130*xl* CW Reproducibility

Figure 5 shows the results of the first reproducibility test on the 3500xL. The results show the majority of the run to run variation of peak heights range from -24% to -50%. All samples showed there was significant difference ($p \geq 0.05$) in peak heights between run 1 and run 2. Peak heights on run 1 were higher than run 2. Between run 1 and run 2 a new pouch of POP4 polymer (same lot number) was loaded to the 3500xL at the beginning of day 2. An electrical discharge error also occurred prior to the processing of run 2. The process was aborted and re-started. The electrical discharge error is usually due to bubbles in the tubing containing the polymer. This could explain the variation between run 1 and run 2. Due to this the reproducibility was repeated. The samples used for the original reproducibility plates were consumed and new samples were selected. Figure 6 shows the results of the repeated reproducibility. The results show the majority of the run to run variation of peak heights range from 1% to -38%. Nine samples showed there was a significant difference ($p \geq 0.05$) and 16 samples showed there was no significant difference ($p \leq 0.05$) in peak heights between run 1

and run 2. For the samples that showed a significant difference the peak heights on run 1 were higher than run 2.

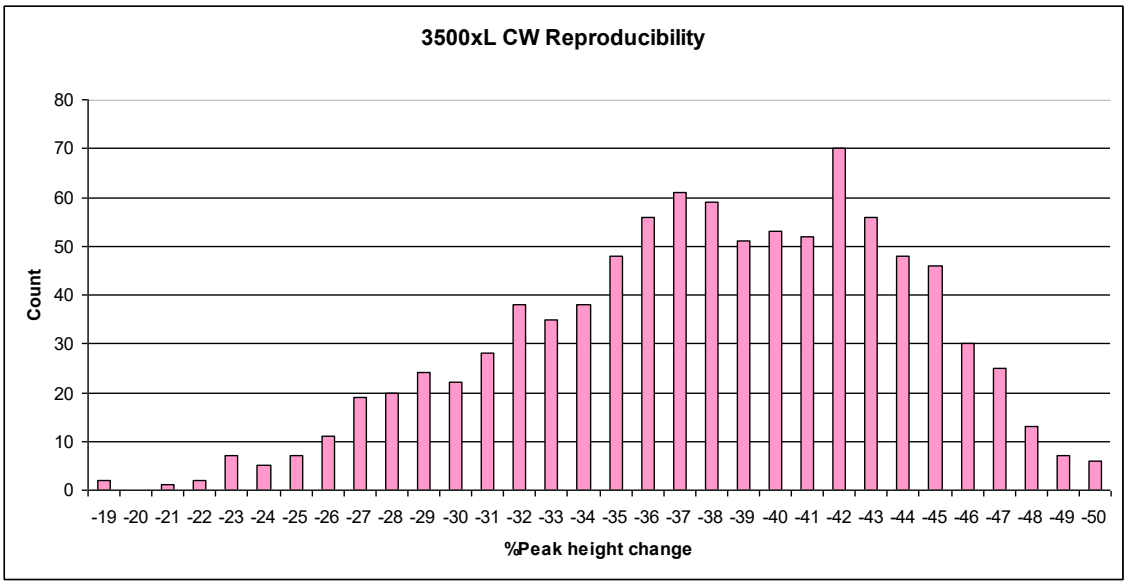

Figure 5 - 3500xL CW Reproducibility

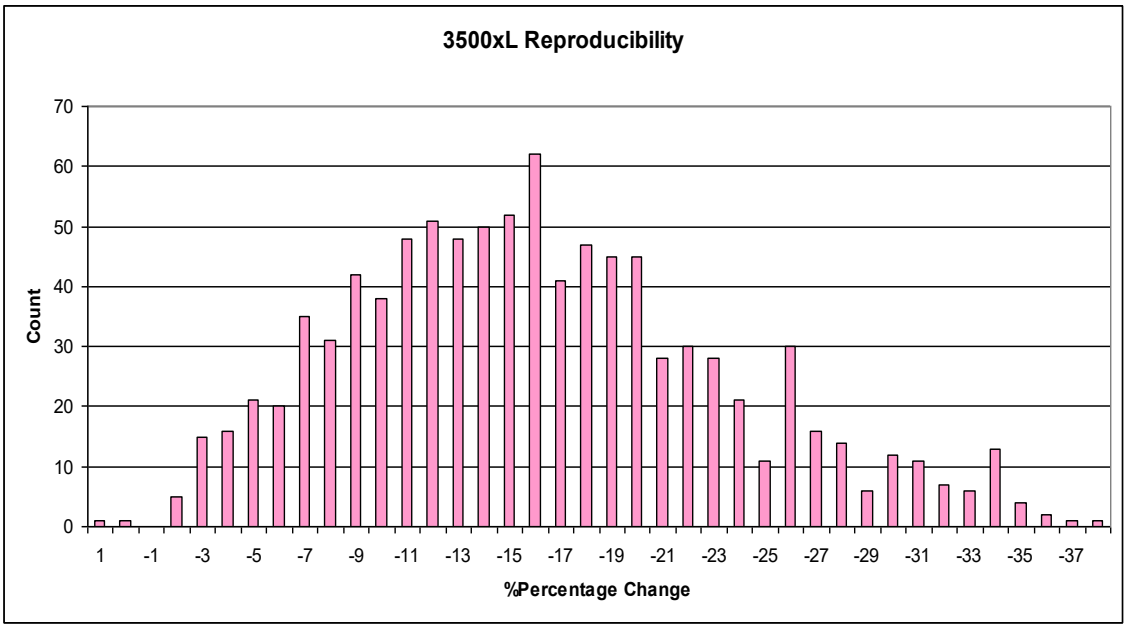

Figure 6 - 3500xL CW Reproducibility repeat

1.1.4 Acceptance Criteria – Repeatability and Reproducibility

All allele designations for samples on the repeatability and reproducibility plates were completely concordant.

Repeatability – the 3500xL showed no significant difference in peak height between runs. Whereas the 3130*xl* showed 2 samples were significantly different in peak height between runs.

Reproducibility – the 3500xL showed 9 samples with a significant different in peak height between runs. Whereas the 3130*xl* showed 5 samples were significantly different.

Based on these results the 3500xL performed slightly better than the 3130*xl* in terms of repeatability and slightly worse in terms of reproducibility.

All allele designations for samples on the repeatability and reproducibility plates were completely concordant.

Repeatability – the 3500xL showed no significant difference in peak height between runs. Whereas the 3130*xl* showed 2 samples were significantly different in peak height between runs.

Reproducibility – the 3500xL showed 9 samples having a significant difference in peak height between runs. Whereas the 3130*xl* showed 5 samples were significantly different.

Based on these results, the 3500xL performed better than the 3130*xl* in terms of repeatability, and worse in terms of reproducibility. Given the results of Section 6.8 Number of Contributors do not support the implementation of the analysis of casework samples amplified with PowerPlex®21, the results of this experiment have not been assessed as to whether the 3500xL has passed or failed.

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